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(54) **Title:** NUCLEOTIDE SEQUENCES ENCODING THE TOMATO LIGHT HYPERSENSITIVE PHENOTYPE, ENCODED PROTEINS AND USES THEREOF

(57) **Abstract**

Nucleotide sequences of the tomato *TDET1 (HP–2)* gene are described, which sequences, if modified, result in a light hypersensitive phenotype. Vectors and uses for the production of transgenic plants are also described and transgenic plants so obtained.

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NUCLEOTIDE SEQUENCES ENCODING THE TOMATO LIGHT HYPERSENSITIVE PHENOTYPE, ENCODED PROTEINS AND USES THEREOF

DESCRIPTION

The present invention relates to nucleotide sequences encoding the tomato light hypersensitive phenotype, encoded proteins and uses thereof.

In particular the present invention relates to nucleotide sequences encoding a protein, whose qualitative or quantitative modification and/or inhibition in plants induces high levels of carotenoids and/or flavonoids and/or chlorophylls, in comparison with wild-type plants; the invention also relates to the use of these nucleotide sequences for the production of engineered plants to be employed in the agro-industrial sector.

Light is a critical environmental signal that controls many aspects of plant growth and development. It is perceived by a sophisticated series of photoreceptors: the phytochromes, which absorb red and far red light, the cryptochromes, which absorb blue and UV-A light wavelengths, and the UV-B receptors (Mustilli and Bowler, 1997). Together with endogenous hormonal signals, these photoreceptors regulate the developmental changes known as photomorphogenesis. Photomorphogenesis is defined as the influence of light on plant development and comprises leaf and chloroplast development and the regulation of photosynthetic apparatus components, by means of the coordinated expression of both nuclear and cytoplasmic genes. Moreover, due to the light response, photoprotectant pigments such as flavonoids are also produced. The modifications occurring during photomorphogenesis have been

characterized by studying light effects on *Arabidopsis* seedling development (von Arnim and Deng, 1996). Light-grown *Arabidopsis* seedlings display short hypocotyls, open and expanded cotyledons and the expression of light-regulated genes which are responsible for flavonoid and chlorophyll biosynthesis (e.g. chalcone synthase, *CHS*; chlorophyll A/B binding protein, *CAB*). Dark-grown seedlings display elongated hypocotyls, closed cotyledons and repression of light-regulated genes.

In higher plants, the phytochromes are encoded by a gene family (e.g. PHYA-E in *Arabidopsis*, Sharrock and Quail, 1989; Clack et al., 1994) and although they are the best characterized photoreceptors, relatively little is known about how the light signals perceived by phytochromes are transduced to the nucleus to activate the various developmental, physiological and molecular responses to light. Recently, biochemical studies using microinjection into cells of the phytochrome deficient *aurea* (*au*) tomato mutant, along with pharmacological studies in photomixotrophic soybean cell cultures, have implicated heterotrimeric G-proteins, cGMP, calcium and calmodulin as intermediates in phytochrome signal transduction pathways (Bowler and Chua, 1994; Mustilli and Bowler, 1997). In parallel, several genetic screens have been developed to identify mutants potentially affected in light signal transduction (Chamowitz and Deng, 1996). Most of the photomorphogenic mutants have been characterised in *Arabidopsis* and can be classified as either insensitive or constitutive mutants. Insensitive mutants display a light-blind elongated phenotype in the light. Some are mutated in the photoreceptors themselves, whilst others are presumed to encode positive regulators of light signal transduction

pathways (Chamovitz and Deng, 1996; Chory et al., 1996; Whitelam and Harberd, 1994). Conversely, constitutive de-etiolated mutants (e.g. *cop/det/fus/cpd*) display light grown morphologies when grown in the dark together, in some cases, with the inappropriate expression of light regulated genes such as *CAB* and *CHS* (Millar et al., 1994; Szekeres et al., 1996). The recessive nature of these mutations suggests that they are loss-of-function and that the wild-type genes are repressors of photomorphogenesis in darkness. However, although epistasis tests with phytochrome-deficient mutants have indicated that they function downstream of phytochrome, they are not specifically mutated in phytochrome signal transduction because many have altered tissue specificities as well as other additional phenotypes not directly related to light (Mayer et al., 1996; Chory and Peto, 1990; Millar et al., 1994; Szekeres et al., 1996). It is therefore not clear how COP/DET/FUS/CPD proteins function in the signal transduction pathways defined biochemically (Bowler and Chua, 1994).

A more targeted approach to identify specific components of signal transduction pathways specific for phytochrome could be the isolation of mutants with altered dynamics of light responses, rather than mutants with constitutive phenotypes in the absence of light. Several such light hypersensitve mutants have already been isolated in tomato (denoted *hp-1*, *hp-2*, *atv*, *Ip*; Kendrick et al., 1994). In particular, the recessive non-allelic *hp-1* and *hp-2* mutants have been characterized by their exaggerated light responsiveness, displaying higher anthocyanin levels (a flavonoid subgroup), shorter hypocotyls and more deeply pigmented fruits than wild-type plants. These mutants were

first identified in 1917 (Reynard, 1956) and in 1975 (Soressi, 1975), respectively. Recently, *hp-1*^{w} (Peters et al., 1989) and *hp-2*^{j} (Van Tuinen et al., 1997) mutants have been isolated and identified as new *hp-1* and *hp-2* alleles, respectively. Because these phenotypes appear to be identical to those obtained by ectopic expression of phytochrome A (*PHYA*) in tomato (Boylan and Quail, 1989), it would appear that the *hp* mutation may affect fairly specifically phytochrome responses. The recessive nature of the mutations, coupled with results from epistasis tests of *hp-1* with the phytochrome deficient tomato mutants *aurea* (*au*), *phyA* (*fri*), and *phyB* (*tri*), have suggested that *HP* genes encode negative regulators of light signal transduction mechanisms, acting downstream of both PhyA and PhyB (Kerckhoffs et al., 1997). The fact that no counterparts of *hp* mutants have been isolated so far in *Arabidopsis*, along with the observation that in tomato anthocyanin production and the expression of photoregulated genes (e.g., *CHS* and *CAB*) is strictly light-dependent, indicates the importance of *hp* mutants for studying phytochrome-dependent signal transduction. Furthermore, microinjection-based studies using the *au* tomato mutant have shown that tomato is an excellent model system to map the role of individual components in the phytochrome activated signalling cascade (Bowler and Chua, 1994). Therefore the identification and characterization of *hp* genes is likely to be very important for studying the regulation of photomorphogenesis in plants.

The authors of the present invention have cloned the tomato *HP-2* gene and have studied at the molecular level the role of the HP-2 protein during the modulation of photomorphogenesis and fruit development. The authors have

found that the tomato *HP-2* gene exhibits high sequence homology with the *Arabidopsis DET1* gene, which belongs to the above described constitutive *COP/DET/FUS* mutant group. Therefore the tomato *HP-2* gene has been renamed *TDET1*.

The authors have used *Solanum lycopersicum* (tomato) species plants, but those skilled in the art will recognize that the cloning could be repeated with no inventive efforts with other plant species, as but not limited to pepper, eggplant, soybean, grape, melon, rice, carrot, spinach, citrus, pomaceae and ornamental species. The authors have cloned and sequenced the gene responsible for the tomato *hp* mutation (*high pigment*), which causes a light hypersensitive phenotype, thus enhancing carotenoid, and/or chlorophyll and/or flavonoid pigment levels. The gene is the first to be identified that causes such a mutant phenotype.

hp mutants potentially have a direct application in the agro-industrial sector, for generating tomato fruits with high carotenoid and/or flavonoid contents. In particular, in tomato fruits of *hp* mutants, a high content of the carotenoid lycopene as well as other carotenoids and flavonoids has been observed(Thompson, 1955; Yen et al., 1997). However, up to now the use of *hp* mutants in the agro-industrial sector, even if bred into various commercial varieties, has been impaired because of the fact that the *hp* mutation generates other undesirable phenotypes, such as reduced internode length and reduced plant vigour.

The cloning of the *TDET1* gene and its use by means of gene transfer technologies offers considerable advantages with respect to conventional breeding techniques. It is possible to transfer genes suitable for agriculture between

different species and to inactivate and/or engineer genes of the same species, even only in specific plant tissues. Furthermore gene transfer is a much more rapid technique than conventional breeding.

The cloning of the *TDET1* gene now allows the production of engineered plants which exhibit all the favourable features of the *hp* mutation, with none of the undesirable side-effects. Such plants could belong to the *Solanum* genus, but those skilled in the art will recognize that, with no inventive effort, it is possible to transfer the gene, or parts thereof, subcloned in suitable expression vectors, into plants belonging to different genera, as e.g. but not limited to pepper, eggplant, soybean, grape, melon, rice, carrot, spinach, citrus, pomaceae and ornamental species.

It has been well recognized that a diet rich in lycopene and other carotenoids, or their administration in the form of pills, produce favourable effects on human health. As a matter of fact, carotenoids have antioxidant properties, ß-carotene is a pro-vitamin A and lycopene is known to be an effective antitumoral agent (Bartley and Scolnik, 1995; Giles and Ireland, 1997; Hoffmann and Weisburger, 1997; Pappalardo et al., 1997; Pool-Zobel et al., 1997; Rock et al., 1997; Sharoni et al., 1997; Stahl and Sies, 1996). Therefore the engineered plants of the present invention can be advantageously utilized as a source of such compounds, either as fresh or processed foods or as nutraceuticals.

Furthermore *hp-2* mutants have high levels of flavonoids such as anthocyanins (Von Wettstein-Knowles, 1968), which are also considered to be excellent antioxidants and which exhibit in some cases antitumoral

properties (Fotsis et al., 1997; Rice-Evans et al., 1997). Furthermore some flavonoids exhibit a role in plant protection against pathogenic agents and UV light irradiation (Shirley,1996).

Therefore the manipulation of TDET1 activity can be used to modify carotenoid and/or flavonoid content in several plant species (e.g., tomato, pepper, eggplant, soybean, grape, melon, rice, carrot, spinach, citrus, and pomaceae) for biotechnological uses in both the biomedical and agro-industrial sectors.

Furthermore the manipulation of *TDET1* gene expression can be used to modify the anthocyanin and carotenoid content in ornamental species for the achievement of new colour variants (Griesbach, 1984).

In addition, because it has been recognized that a high content of carotenoids improves resistance to Norflurazon-type herbicides, a further application of the *TDET1* gene is the production of transgenic plants by selection using herbicides rather than antibiotic compounds (Misawa et al., 1993).

Furthermore in the same plant it is possible to combine a modified TDET1 activity with mutations such as *rin*, *nor* and *Nr*, which interrupt the fruit ripening process, or with biosynthetic genes of the carotenoid biosynthesis pathway (Bartley and Scolnik, 1995), to obtain varieties exhibiting new qualitative characteristics for agro-industry.

The attainment of an *hp* mutant phenotype by means of a biotechnological approach can be carried out advantageously by means of the inhibition of TDET1 activity. Currently the best method for reducing gene expression is through the introduction, by gene transfer,

of the antisense sequence of the same gene or part thereof under the control of appropriate regulatory sequences. The use of such techniques in plants has been carried out in several existing examples (Oeller et al., 1991; Penarrubia et al., 1992), but those skilled in the art will recognize that alternative techniques can be used, without departing from the scope of the present invention.

Furthermore, by using specific vectors such as, for example, but not limited to, pE8mGFP4, which contains regulatory sequences of the tomato *E8* gene (Fig. 9) (Deikman and Fischer, 1988), the inactivation of the *TDET1* gene can be specifically modulated in the tomato fruit. Those skilled in the art will recognize that other regulatory sequences, e.g., originating from the polygalacturonase gene (*PG*) (Nicholass et al., 1995), can be used in the place of the *E8* gene promoter to obtain specific tissue modulation of *TDET1* gene expression.

Within the scope of the present invention the term "light hypersensitive phenotype" means reduced plant growth associated with high levels of carotenoids and/or chlorophylls and/or flavonoids.

The term "protein or functional parts thereof, responsible for the light hypersensitive mutant phenotype" means an amino acid sequence which, if structurally or otherwise altered, induces a light hypersensitive phenotype in a plant.

Therefore one object of the present invention is a nucleic acid comprising a nucleotide sequence encoding a protein, or functional parts thereof, which, if modified, is responsible for the light hypersensitive mutant phenotype in *Solanum lycopersicum* plants, said phenotype including reduced plant growth associated with high levels

of carotenoids and/or chlorophylls and/or flavonoids. Nucleic acids encoding proteins which are homologous to proteins of the *Arabidopsis* COP/DET/FUS family, which when modified result in a light hypersensitive phenotype, are within the scope of the present invention.

Preferably the nucleic acid comprises the nucleotide sequence encoding the TDET1 protein, or functional parts thereof. More preferably the nucleic acid comprises a nucleotide sequence encoding the protein having the amino acid sequence of SEQ ID No. 2 or functional portions thereof. More preferably the nucleic acid has a nucleotide sequence comprised in SEQ ID No. 1, more preferably from nt. 149 to nt. 1720. Alternatively the nucleic acid has a nucleotide sequence complementary to SEQ ID No. 1, or parts thereof.

In one aspect of the invention the nucleic acid of SEQ ID No. 1 comprises a mutation which is able to induce the light hypersensitive phenotype; preferably at least a C→T substitution in position 1640; alternatively the nucleic acid of SEQ ID No. 1 is deleted at least from nt. 1581 to nt. 1589.

A further object of the present invention is an expression vector including, under the control of an active and inducible plant promoter, the nucleic acid of the invention. Preferably the promoter is active only in some plant organs, more preferably in fruits. Preferred vectors are able to drive the transcription of an antisense RNA, for example pBIN-E8-HP2-AS1 and pBIN-E8-HP2-AS2.

A further object of the invention is the use of the vectors of the invention for producing transgenic plants which comprise the nucleic acid, under the control of specific regulating sequences, preferably in preselected

plant organs. Plants can belong to pepper, eggplant, soybean, grape, melon, rice, carrot, spinach, citrus, pomaceae and ornamental species.

A further object of the invention is a transgenic plant, or parts thereof, achievable by transformation with the vectors of the invention. Plants can belong to pepper, eggplant, soybean, grape, melon, rice, carrot, spinach, citrus, pomaceae and ornamental species.

Plant genetic transformation techniques are known to those skilled in the art and comprise, but are not limited to transformation by Agrobacterium, electroporation, microinjection, or bombardment with DNA coated particles(Christou, 1996).

In a further aspect the invention includes a protein, or functional parts thereof, whose modification is responsible for the light hypersenstive phenotype in *Solanum lycopersicum* plants. Proteins homologous to the *Arabidopsis* COP/DET/FUS family are within the scope of the present invention, provided that, when modified, they result in a light hypersensitive phenotype. Preferably the protein comprises the amino acid sequence of SEQ ID No. 2 or parts thereof.

In one aspect of the invention the protein comprises a modification which is able to induce the light hypersensitive phenotype; preferably at least a modification in its C-terminal portion; more preferably a replacement of proline at position 498, most preferably serine as a substitute for proline; alternatively a deletion of at least one amino acid, preferably at least of three amino acids, more preferably from aa. 478 to aa. 480 of SEQ ID No. 2.

The present invention will be described by reference to explanatory, but not limiting, examples, wherein reference will be made to the following figures:

Figure 1. Phenotypes of wild type, *hp-2*, and *hp-2^j* tomato seedlings grown in the presence or in the absence of light. a) Hypocotyl length of seedlings grown in the absence (Dark) and in the presence of light (Light). b) Content of anthocyanins in the hypocotyls (dashed bars) and in the cotyledons (empty bars) of seedlings grown in the light. Data represent average values from ten seedlings. Following a three day pre-germination period in darkness, seedlings were grown either in darkness (Dark) or in a 16 hour light, 8 hour dark photoperiod (Light) for 6 days.

Figure 2. *TDET1* (*HP-2*) cDNA nucleotide sequence (SEQ ID NO. 1). The sequence encoding the TDET1 protein is depicted in capital letters. The sequence of the RFLP CT151 marker covers nt. 830 to nt. 2000.

Figure 3. Amino acid sequence of the TDET1 (HP-2) protein (SEQ ID No. 2). Amino acids are depicted in the standard one letter code for amino acids.

Figure 4. Exon-intron structure of the tomato *TDET1* (*HP-2*) gene and sites of mutations in *hp-2* and *hp-2^j* mutant alleles. Stippled boxes indicate exons. Exons correspond to the following sequences of SEQ ID No. 1 (Figure 2): Exon 1: nt. 149 to nt. 220; Exon 2: nt. 221 to nt. 648; Exon 3: nt. 649 to nt. 877; Exon 4: nt. 877 to nt. 1012; Exon 5: nt. 1013 to nt. 1081; Exon 6: nt. 1082 to nt. 1138; Exon 7: nt. 1139 to nt. 1219; Exon 8: nt. 1220 to nt. 1384; Exon 9: nt. 1385 to nt. 1480; Exon 10: nt. 1481 to nt. 1580; Exon 11: nt. 1581 to nt. 1720; b) Donor and acceptor splice sites of intron 10 from the wild type and the *hp-2* mutant. Brackets indicate splice sites, dots indicate internal intron

sequence (not shown), and amino acids are shown in the one letter code. The asterisk in the *hp-2* sequence denotes the site of the mutation.

Figure 5. Alignment of the amino acid sequences of *S. lycopersicum* TDET1, *Arabidopsis* DET1, and deduced mammalian EST amino acid sequences with homology to DET1 (determined using Clustal method; Higgins and Sharp, 1988). The putative bipartite NLS is overlined. The amino acids missing in the *hp-2* mutant are indicated with asterisks, and the amino acid substitution in *hp-2^j* is denoted by a plus sign. The mammalian sequences are a compilation of derived amino acid sequences from mouse and human ESTs (GenBank accession numbers AA756238, AA236057, AA050184, and W64359). Boxed residues indicate conserved amino acids, dashes indicate arbitrary insertions.

Figure 6. Effect of cytokinins on wild type tomato seedlings.(a) and (b) Hypocotyl length (cm) and anthocyanin content (per mg fresh weight [FW]), respectively, of wild type tomato seedlings grown in the presence of 0, 1, 5, 20, 100, and 500 µg/L benzoaminopurine. Seedlings were grown at 25 °C for 5 days in absolute darkness (Dark) or in a 16-hr light 8-hr dark photoperiod (Light). In (a) values are the mean of 10 seedlings. Highly similar results were obtained with the cytokinin zeatin (data not shown).

Figure 7. Phenotype of the *au hp-2* double mutant phenotype. Hypocotyl length (cm) and anthocyanin accumulation (per seedling) in wild type, *au*, *hp-2*, and the *au hp-2* double mutant are shown. Seedlings were grown at 25 °C for 6 days in a 16-hr light 8-hr dark photoperiod. Values are the mean of 15 seedlings, and the experiments were repeated three times.

Figure 8. Gene expression in wild type, *hp-2*, and *hp-2^j* seedlings. Seedlings were grown at 25 °C for 5 days in absolute darkness (D), followed by 2 days in continuous white light (L). The RNAs were extracted from whole seedlings (dark) or from cotyledons (C) and hypocotyls (H) (light). Modifications in gene expression in *hp-2* and *hp-2^j* seedlings compared with wild type seedlings (wt) are shown to be principally light-dependent. Ten microgram samples of total RNA were loaded on gels and analyzed for expression of *CHS1* (*CHS*), *CAB6* (*CAB*), *PR1-1b1* (*PR*) genes following RNA gel blotting. 30S rRNA is shown as a control for loading.

Figure 9. Binary plasmids to be transferred to plants.

Materials and Methods

Plant material and growth conditions

The *hp-2* and *hp-2^j* exaggerated photoresponse mutants, the *au hp-2* double mutant, and the corresponding wild type tomato seeds (*Solanum lycopersicum* cv Money Maker) were kindly provided by R. E. Kendrick and M. Koornneef (Wageningen Agricultural University, the Netherlands). Seeds were surface sterilized and directly sown in magenta boxes (Sigma) containing 4.3 g/L Murashige-Skoog salts (Sigma) and 0.8 % agar. After 2 days pregermination in darkness, seedlings were grown at 25° C either in a 16 hour light, 8 hour dark photoperiod or in continuous dark, as appropriate. For cytokinin treatment, seeds were sown in the presence of different concentrations of benzylaminopurine (Sigma).

Anthocyanin assay

Anthocyanins were extracted from cotyledons, hypocotyls, and whole seedlings with 0.5 mL acidified (1% HCl) methanol for 48 hr in darkness with shaking. The

extracts were separated by the addition of 0.4 mL of H_2O and 1 mL of chloroform, followed by centrifugation for 5 min at 3,000 rpm. The absorbance of the upper phase was determined spectrophotometrically at 535 nm (A_{535}), and the anthocyanin content was calculated as (A_{535})/mg fresh weight or (A_{535})/seedling.

Carotenoid and Chlorophyll assay

In order to determine the content of carotenoids and chlorophyll in the tomato fruit, pericarp sections of unripe (25 days after pollination) or ripe (50 days after pollination) fruit were weighed and incubated for 48 hours at 65°C in DMSO (dimethyl sulfoxide) in the absence of light. The contents of carotenoids and chlorophylls were determined by HPLC and values are reported as µg/g fresh weight.

DNA and RNA Extraction

DNA and RNA extractions from leaf samples were carried out according to Dellaporta et al. (1983) and Verwoerd et al. (1989), respectively.

RNA gels (10 µg per lane) were blotted onto Hybond N+ membranes (Amersham) and hybridized with random primed probes (see below). Hybridization was performed for 24 hr at 50°C in phosphate buffer (7% SDS, 0.5M Na_2HPO_4, pH 7.2, 1mM EDTA), followed by 20-min washes in 40mM $NaPO_4$ pH 7.2, 1% SDS, and 1mM EDTA. Probes were the tomato chalcone synthase gene *CHS*1 (O'Neill et al., 1990), chlorophyll a/b binding protein gene *CAB*6 (Piechulla et al., 1991) and pathogenesis-related protein gene *PR-1b1* (Tornero et al., 1997). All RNA gel blots were repeated at least twice and using different samples.

Isolation and sequence of genomic clones, cDNAs and PCR products

5′ amplification of cDNA was performed using the RACE system (Gibco/BRL) on total tomato leaf RNA with the oligonucleotide 5′-CATCAACACTGCCAAAC-3′(SEQ ID No. 3), derived from the CT151 RFLP marker sequence. A 0.8-kb Polymerase Chain Reaction (PCR) product, containing the 5' end of the *TDET1* cDNA, was obtained after two different amplification reactions with Taq1 polymerase (Perkin Elmer) using two CT151-derived nested primers: 5′-GAAAGCAGCCGTTGCT-3′ (SEQ ID No. 4) and 5′-AGTTCATCATCTTCACGGC-3′ (SEQ ID No. 5), with the provided anchor primer (Gibco/BRL) The 0.8kb PCR fragment was directly sequenced on both strands with thermosequenase (Amersham).

Total cDNA from wild type tomato (cv. Money Maker) and the corresponding *hp-2* and *hp-2^j* mutants were obtained by reverse transcription using avian myeloblastosis virus reverse transcriptase (Promega) of poly(A) mRNA isolated from leaves using oligo(dT) Dynabeads (Dynal). From these samples the *TDET1* (*HP-2*) cDNA sequence was PCR amplified with the specific oligonucleotides:
5′-GTATGATTCACTAGTTTAATGCTGCTGAAAG-3′ (SEQ ID No. 6) and
5′-CCCATACTAGTCGTCTTGGCACTCTATCAAG-3′ (SEQ ID No. 7), using the Expand High Fidelity system (Boehringer Mannheim). Subsequently the amplification product was subcloned in pBluescript as a SpeI fragment and 4 independent clones were sequenced on both strands.

Tomato genomic libraries in λ–DASH and λ–FIXII (kindly provided, respectively, by Jim Giovannoni, Texas A&M University, College Station, TX, and the Tomato Genome Center, Rehovot, Israel) were screened using standard methods (Sambrook et al., 1989) with a ^{32}P-labelled CT151 fragment as a probe. Overlapping fragments from different

recombinant phages were subcloned in pBluescript SK+ for sequencing of both strands.

The 5'-GAAGGTAATTTTATATTAAACATAGAATAGA-3' (SEQ ID No. 8) and 5'-GTGATTTCTAGGTTGATTTCAATCTAGA-3' (SEQ ID. No. 9) oligonucleotides were used to amplify the *HP-2* gene 3' sequence from genomic DNA of the *hp-2* mutant. The 1,300 bp amplification product was directly sequenced with the primer 5'-CAAATCGGTAACATAT-3' (SEQ ID No. 10) using a Thermosequenase kit (Amersham).

Binary plasmids for plant transformation

DNA fragments containing the tomato fruit-specific E8 promoter (Deikman and Fischer, 1988) were PCR amplified from *S. lycopersicum* total DNA with the following nucleotides: 5'-GGGGAAGCTTTTTCACGAAATCGGCCCTTA-3' (SEQ ID No. 11) and 5'-CCCGGATCCTTCTTTTGCACTGTGAATGATTAG-3' (SEQ ID No. 12). The 1.2kb amplification products were subcloned as HindIII-BamHI fragments in the binary expression vector pmGFP4, derived from pBI121 (Bevan, 1984;Jefferson et al., 1987; Haseloff et al., 1997) in place of the 35S promoter, obtaining the plasmid pE8mGFP4. The mGFP4 gene was then excised and the inverted or complementary sequence of the *HP-2* gene, or parts thereof, was inserted, as BamHI-SacI fragments, obtaining the plasmid pBIN-E8-HP2-AS1 and pBIN-E8-HP2-AS2, respectively (Figure 9).

Results

hp-2 mutant phenotypes

hp-2 mutant seedlings exhibited an exaggerated light response with respect to wild type seedlings. Particularly, hypocotyl length was reduced (Figure 1a) and the content of anthocyanin pigments in the cotyledons was higher than in the wild type (Figure 1b). Such phenotypes were strictly dependent on the presence of light; in fact in the absence

of light the length of hypocotyl was not considerably different to the wild type (Figure 1a) and anthocyanins were not produced (data not shown). The *hp-2^j* mutant exhibited a stronger phenotype than the *hp-2* mutant, with reference both to hypocotyl length and the content of anthocyanins (Figure 1).

Fruits obtained from plants of *hp-2* and *hp-2^j* mutants also exhibited exaggerated light responses. In the unripe fruits from *hp-2* and *hp-2^j* mutants the content of chlorophyll was about five times higher than in fruits from wild-type plants, whereas in mature fruits the total content of carotenoids was about twice that of fruits from wild-type plants (Table 1).

Table 1

Chlorophyll and carotenoid content of tomato fruits from wild type, *hp-2* and *hp-2^j* mutants.

Genotype	Total Chlorophylls (ripe fruit) µg/g fresh weight	Total Carotenoids (unripe fruit) µg/g fresh weight
wild type (Money Maker)	20	60
hp-2	90	124
hp-2^j	115	137

The Phenotype of the Tomato *hp-2* Mutant is Caused by Mutation in *DET1*

The *hp-2* mutation has been mapped previously using restriction fragment length polymorphism (RFLP) analyses of a segregating population derived from a cross between the *hp-2* mutant (*S. lycopersicum*) and *S. pennellii*. Mapping data with a second backcross (BC-2) population indicated a position close to the centromere of chromosome 1, within a

cluster of several RFLP markers (Balint-Kurti et al., 1995; Van Tuinen et al., 1997; Broun and Tansksley, 1996). The authors of the present invention have found that the CT151 RFLP marker (Tanksley et al., 1992), included in this cluster, had high homology with the *Arabidopsis DET1* gene, mutations in which were previously identified as causing the deetiolated phenotype of *det1* mutants.

Comparison of CT151 with *Arabidopsis DET1* revealed that CT151 has homology to the 3' end of *DET1*. The authors of the present invention isolated a full-length cDNA encoding *TDET1* using 5' rapid amplification of cDNA ends (Loh et al., 1989). Subsequently, the *TDET1* gene was isolated from genomic libraries in λ-DASH and λ-FIXII. Alignment of the *TDET1* cDNA and genomic sequences revealed the presence of 10 introns, which is similar to the number found in *Arabidopsis* (Figure 4a). Nine introns are located in the same positions as those of the *Arabidopsis DET1* gene, whereas intron 2 of *TDET1* is not present in the *Arabidopsis* homolog (Figure 4a) (Pepper et al., 1994). Comparative protein sequence analysis between *Arabidopsis* DET1 and TDET1 show 81.3% similarity and 74.8% identity (Figure 5). No major differences are present between the sequences, except for a small deletion of 16 amino acids at the centre of *TDET1*, suggesting that the tomato and *Arabidopsis* genes are true homologs. A *DET1* homolog is not present in the yeast genome or in any prokaryotic genome sequenced to date (data not shown). However, mouse and human expressed sequence tags (ESTs) with homology to *DET1* have been identified (Figure 5), as well as a Drosophila homolog.

Considering the presence of a nuclear localisation signal (NLS) (Robbins et al., 1991) in the *Arabidopsis* DET1

amino acid sequence, and observing that the DET1-GUS chimeric protein is localised in the nucleus (Pepper et al., 1994), DET1 in *Arabidopsis* may be responsible for the transcriptional repression of photomorphogenesis in the absence of light. Considering the homology with HP-2 it is possible for the latter to have an analogous function in tomato. The TDET1 protein has been found to be localized in the nucleus (data not shown).

TDET1-encoding cDNAs from the wild type, and *hp-2* and *hp-2^j* mutants were amplified with specific primers from leaf mRNA. Amplified fragments were subcloned in pBluescript and 4 independent clones were sequenced on both strands. In *hp-2* the authors identified a substitution involving T in the place of C in exon 11 (nt. 1640) that causes a replacement of proline by serine in the protein C-terminal region (aa. 498) (Figure 4a). In the *hp-2* mutant the authors identified an alternative splicing site for intron 10, which causes a three amino acid deletion (Gly, Pro, Glu) at the start of exon 11, in the second NLS domain of the protein (aa. 478-480) (Figure 4a). To find the mutation site which was responsible for the alternative splicing, the authors sequenced the 3'end of the *TDET1* gene from the *hp-2* mutant, using a fragment obtained by PCR amplification of genomic DNA. A replacement of AG by TG in the consensus 3' splice junction of intron 10 was identified (Figure 4b). These results demonstrate that the "high pigment" phenotype of the *hp-2* and *hp-2^j* tomato mutants is caused by mutation of the *TDET1* gene mutation.

Sequencing of the *TDET1* cDNAs derived from the *hp-2* mutant showed that the mutation produces two different splicing products from intron 10 (Figure 4b), suggesting the presence of a limited amount of wild-type TDET1 protein

and that the *hp-2* mutant is not an inactive allele. This is consistent with the observation that the *hp-2* mutant allele is weaker than the *hp-2^j* allele (Figure 1 and Table 1). Whether the *hp-2^j* mutation is null awaits further analysis, although the mutated proline residue in the *hp-2^j* mutant is conserved in both the plant sequences. However, because the *hp-2* mutation is situated in the C-terminal domain of TDET1, as is the mutation in the *Arabidopsis det1-5* mutant allele, which has a clearly de-etiolated phenotype (Pepper et al., 1994), it is apparent that the *hp-2* mutant phenotypes are not comparable with the *det1* mutant phenotype in *Arabidopsis*. Furthermore, clearly weak alleles of *det-1* in *Arabidopsis*, eg. *det1-1*, display visible dark phenotypes.

Comparison of Tomato *hp-2* and *Arabidopsis det1* Mutants

As is clear from the results presented, *hp-2* mutants are phenotypically different from *det1* mutants. Most conspicuously they do not display dark phenotypes, such as reduced hypocotyl length, opened apical hooks, or enlarged cotyledons, whereas these were selection criteria for the isolation of *det* and *cop* in *Arabidopsis* (Chory et al., 1989; Deng et al., 1991). Conversely, *det1* mutants can even develop true leaves and floral buds in prolonged darkness. No such phenotypes can be observed in *hp* tomato seedlings grown in the dark.

Arabidopsis det1 mutants are also characterized by high level expression of light-regulated genes such as *CHS* and *CAB* in the dark, whereas in the light, *CHS* and *CAB* gene expression are similar to that observed in wild-type plants (Chory et al., 1989). To examine the effects of *hp-2* and *hp-2^j* mutations on gene expression, the authors have performed RNA gel blot analysis of light-regulated gene

expression using *CHS* and *CAB* gene fragments as probes. In agreement with the weak phenotypes of *hp-2* and *hp-2^j* mutant seedlings grown in darkness, no dramatic alteration of *CHS* and *CAB* gene expression was observed. Nonetheless, *CHS* and *CAB* mRNA levels were slightly higher in the *hp-2^j* allele when compared with *hp-2* and wild-type seedlings (Figure 8). Interestingly, *CHS* mRNA appeared to be of a slightly smaller size than that found in light-grown material, perhaps indicating a differential light-dependent splicing. Consistent with the exaggerated photoresponsiveness of *hp-2* and *hp-2^j* mutants, *CHS* mRNA levels were significantly enhanced compared with wild-type seedlings following light irradiation for 48 hr (Figure 8). *hp-2^j* seedlings contained higher expression levels of *CHS* than *hp-2*, and *CHS* mRNA was particularly abundant in hypocotyls. In contrast to *CHS*, *CAB* mRNA levels were higher in cotyledons than in hypocotyls, and were slightly lower in light-exposed *hp-2* and *hp-2^j* mutant seedlings when compared to the wild type (Figure 8). In summary therefore, in tomato *hp-2* mutants dark expression of *CHS* and *CAB* genes is only very slight and deregulation of gene expression is essentially light-dependent. This is in strong contrast to the *CHS* and *CAB* gene deregulation characteristics of *Arabidopsis det1* mutants (Pepper et al., 1994).

det1 seedlings have been also reported to display a strongly enhanced expression of stress-related genes such as those encoding pathogenesis-related (PR) proteins and glutathione reductase (Mayer et al., 1996) To examine whether this was also the case in *hp-2* and *hp-2^j* mutants, RNA gel blots were hybridized with a probe encoding tomato *PR-1b1* (Tornero et al., 1997). Although hypocotyls of *hp-2* seedlings reproducibly displayed *PR-1b1* gene expression at

slightly higher levels compared to wild-type seedlings, this effect was very weak compared to that observed in *Arabidopsis det1* mutants and has never been observed in *hp-2^j* mutants (Figure 8).

Despite these above-mentioned differences, plastid development in darkness in the cotyledons of *hp-2* and *hp-2^j* seedlings was found by the authors to be similar to that observed in *Arabidopsis det1* mutants (data not shown; Chory et al., 1989).

Cytokinin can Phenocopy the *hp* Mutant

Previous observations in *Arabidopsis* have shown that a *det1* phenotype can be phenocopied by the exogenous application of cytokinin (Chory et al., 1994).

To determine the effects of cytokinin treatment in tomato, the authors treated wild-type seedlings with different concentrations of cytokinin in the dark and in the light (Figure 6). Cytokinin was found not to phenocopy a *det1* mutation in tomato. In dark-grown seedlings, although hypocotyls are shorter in the presence of cytokinin, apical hook opening, cotyledon expansion, and anthocyanin biosynthesis were not observed (Figure 6), even after prolonged periods (up to three weeks) (data not shown). However, in the light, cytokinin can phenocopy the *hp* mutation: seedlings displayed shorter and thicker hypocotyls and accumulated high levels of anthocyanin (Figure 6).

These results therefore indicate that at least as far as the effects of cytokinin are concerned, the *hp* mutation in tomato is equivalent to the *det1* mutation in *Arabidopsis*. Such a supposition is consistent with the fact that no constitutive de-etiolated mutants have heretofore been identified in tomato and *CHS* gene expression and

consequent biosynthetic build-up of anthocyanin pigments is strictly dependent on the light, whereas in *Arabidopsis* anthocyanins can be produced also in the absence of light (Mustilli and Bowler, 1997).

Double Mutant Analyses

Double mutant analysis with *det1* and phytochrome-deficient mutants in *Arabidopsis* indicate that the *det1* mutation is completely epistatic to photoreceptor mutations (Chory, 1992). This has been interpreted as meaning that DET1 acts downstream of phytochrome. To determine the relationship between TDET1 and phytochrome function, we examined the effects of the *hp-2* mutation in the *aurea* mutant background, a phytochrome chromophore-deficient mutant (Terry and Kendrick, 1996). In contrast to its counterpart double mutant in *Arabidopsis*, *au hp-2* is similar to the single mutant *au*. For example, hypocotyls are elongated and anthocyanin accumulation is limited (Figure 7). Although in genetic terms this result therefore infers that *au* is epistatic to *hp-2*, the authors nonetheless consider it more likely that TDET1 acts downstream of phytochrome, as proposed for *Arabidopsis*, but that its activity as a negative regulator is strictly dependent upon the presence of active phytochrome. This requirement is clearly not observed in *Arabidopsis* (Chory, 1992). The small but significant reduction in hypocotyl length and the small increase in anthocyanin found in the *au hp-2* double mutant compared with *au* (Figure 7) is likely therefore to be a result of hypersensitivity caused by the *hp-2* mutation towards the low amounts of functional phytochrome present in the *au* mutant, which is estimated to be about 3% wild-type levels (Adams et al., 1989; Parks et al, 1987). Therefore the *hp-2* phenotype in tomato is

strictly dependent on the presence of phytochrome, whereas in *Arabidopsis* the *det1* mutation is independent of the presence or absence of functional photoreceptors.

The absence of de-etiolated mutants in tomato and the strict light- and phytochrome- dependence of the *hp* mutant phenotype may suggest that in tomato TDET1 function is either redundant or that the signal transduction pathways regulating its activity operate in different ways in the two plants (Mustilli and Bowler, 1997). Because by Southern analysis the authors have shown that in tomato, like in *Arabidopsis* (Pepper et al., 1994), only one *DET1* homologous gene is present (data not shown), the latter hypothesis seems to be more likely.

Based upon the author's findings, it is likely that only a few key regulators will be found to be responsible for controlling light responses in all higher plants. Therefore it would be very interesting to clone other tomato genes encoding proteins homologous to COP/DET/FUS and examine whether modification of their activity also results in light hypersensitive phenotypes.

Binary plasmids for modulation of *TDET1* activity

In order to generate tomato plants with an *hp-2* phenotype specifically in the fruit, the authors constructed plasmids which are able to produce the *TDET1* antisense RNA sequence, or portions thereof, only in the fruit by using the regulatory sequences of the tomato *E8* gene (Figure 9).

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(A) NAME: Stazione Zoologica "Anton Dohrn"
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(F) POSTAL CODE (ZIP): 80121

(ii) TITLE OF INVENTION: Nucleotide sequences encoding the tomato light hypersensitive phenotype, encoded proteins and uses thereof

(iii) NUMBER OF SEQUENCES: 12

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2000 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA to mRNA
(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:149..1720
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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TTACAGCTTC TCTTCTAATT AATTGATGTA CATTCAGATG TTAAAAAAAA AAAAAAAAAA 2000
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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 524 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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Glu Asn Val Val Pro Ser Tyr Thr Ile Tyr Asp Val Glu Cys Pro Asp
35 40 45

His Ser Phe Arg Lys Phe Thr Asp Asp Gly Leu Tyr Phe Val Ser Phe
50 55 60

Ser Arg Asn His Gln Asp Leu Val Val Tyr Arg Pro Thr Trp Leu Thr
65 70 75 80

Phe Ser Cys Lys Glu Glu Asp Cys Asp Thr His Asp Leu Pro Leu Lys
85 90 95

Ala Arg Lys Phe Glu Ser Phe Phe Thr Gln Leu Tyr Ser Val Thr Leu
100 105 110

Ala Ser Ser Gly Glu Leu Ile Cys Lys Asp Phe Phe Leu Tyr Met Glu
115 120 125

Ser Asn Gln Phe Gly Leu Phe Ala Thr Ser Thr Ala Gln Ile His Asp
130 135 140

Ala Pro Pro Thr Gly Gly Ala Ile Gln Gly Val Pro Ser Val Glu Lys
145 150 155 160

Ile Thr Phe His Leu Leu Arg Leu Val Asp Gly Ala Ile Leu Asp Glu
165 170 175

Arg Val Phe His Asn Asp Tyr Val Asn Leu Ala His Ser Ile Gly Ala
180 185 190

Phe Leu Tyr Asp Asp Leu Leu Ala Ile Val Ser Leu Arg Tyr Gln Arg
195 200 205

Ile His Ile Leu Gln Ile Arg Asp Ser Gly Asp Leu Val Asp Val Arg
210 215 220

Ala Ile Gly Glu Phe Cys Arg Glu Asp Asp Glu Leu Phe Leu Asn Ser
225 230 235 240
Asn Ser Gln Val Leu Val Asn His Val Gly Asn Gly Phe His His Ser
245 250 255
Leu Pro Gln Ser Glu Thr Ser Phe Leu Ser Gly Ile Lys Gln Arg Leu
260 265 270
Leu Ser Tyr Ile Phe Arg Gly Ile Trp Asn Glu Ala Asp Gln Thr Met
275 280 285
Arg Val Gln Cys Leu Lys Lys Lys Phe Tyr Phe His Phe Gln Asp Tyr
290 295 300
Ile Asp Leu Ile Ile Trp Lys Val Gln Phe Leu Asp Arg His His Leu
305 310 315 320
Leu Ile Lys Phe Gly Ser Val Asp Gly Gly Val Ser Arg Asn Ala Asp
325 330 335
Ile His Pro Ser Phe Phe Ala Val Tyr Asn Met Glu Thr Thr Glu Ile
340 345 350
Val Ala Phe Tyr Gln Asn Ser Ala Asp Glu Leu Tyr Phe Leu Phe Glu
355 360 365
Leu Phe Ser Asp His Phe His Val Ser Ser Lys Ser Ser Leu His Met
370 375 380
Asn Phe Met Ser Ser His Ser Asn Asn Ile His Ala Leu Glu Gln Leu
385 390 395 400
Arg Cys Thr Lys Asn Lys Ala Thr Asn Phe Ser Gln Phe Val Lys Lys
405 410 415
Met Met Ala Ser Leu Pro Cys Ser Cys Gln Ser Gln Ser Pro Ser Pro
420 425 430
Tyr Phe Asp Gln Ser Leu Phe Arg Phe Asp Glu Lys Leu Ile Ser Ala
435 440 445
Ile Asp Arg His Arg Gln Ser Thr Asp His Pro Ile Lys Phe Ile Ser
450 455 460
Arg Arg Gln Pro Asn Ile Leu Lys Phe Lys Met Lys Pro Gly Pro Glu
465 470 475 480
Ala Gly Ser Thr Asp Gly Arg Thr Lys Lys Ile Cys Ser Phe Leu Phe
485 490 495
His Pro Ile Leu Pro Leu Ala Leu Ser Val Gln Gln Thr Leu Phe Leu
500 505 510

Gln Ala Ser Val Val Asn Ile His Phe Arg Arg *
515 520

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CATCAACACT GCCAAAC 17

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GAAAGCAGCC GTTGCT 16

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

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(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

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(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GAAGGTAATT TTATATTAAA CATAGAATAG A 31

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GTGATTTCTA GGTTGATTTC AATCTAGA 28

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CAAATCGGTA ACATAT 16

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GGGGAAGCTT TTTCACGAAA TCGGCCCTTA 30

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CCCGGATCCT TCTTTTGCAC TGTGAATGAT TAG 33

CLAIMS

1. Nucleic acid comprising a nucleotide sequence encoding a protein, or functional portions thereof, which, if altered, is responsible for the light hypersensitive mutant phenotype in *Solanum lycopersicum* plants, said phenotype comprising a reduced growth of the plant associated with high levels of carotenoids and/or cholophylls and/or flavonoids.

2. Nucleic acid according to claim 1 encoding a tomato protein homologous to at least one of the *Arabidopsis* COP/DET/FUS family proteins, provided that it is responsible for the light hypersensitive mutant phenotype.

3. Nucleic acid according to claim 2 comprising the nucleotide sequence encoding the TDET1 (HP-2) protein, or functional portions thereof.

4. Nucleic acid according to claim 3 comprising a nucleotide sequence encoding the protein having the amino acid sequence of SEQ ID No. 2, or functional portions thereof.

5. Nucleic acid according to claim 4 comprising a nucleotide sequence comprised in SEQ ID No. 1.

6. Nucleic acid according to claim 5 wherein the nucleotide sequence comprised in SEQ ID No. 1 is the sequence from nt. 149 to nt. 1720, or portions thereof.

7. Nucleic acid comprising a nucleotide sequence complementary to SEQ ID No. 1 or portions thereof.

8. Nucleic acid according to claim 3 or 4 comprising a mutation which is able to induce the light hypersensitive phenotype.

9. Nucleic acid according to claim 8 wherein the SEQ ID No. 1 comprises at least a T as a substitute for C at position 1640.

10. Nucleic acid according to claim 8 wherein the SEQ ID No. 1 comprises at least a nucleotide deletion from nt. 1581 to nt. 1589.

11. Expression vector comprising the nucleic acid according to any one of preceding claims under the control of a promoter that is active in plants.

12. Vector according to claim 11 wherein said promoter is selectively active only in a few plant organs.

13. Vector according to claim 12 wherein said promoter is active in the fruits.

14. Vector according to any one of preceding claims 11-13 which is able to control the transcription of an antisense RNA.

15. Vector according to claim 14 wherein said vector is the pBIN-E8-HP2-AS1 vector or the pBIN-E8-HP2-AS1 vector.

16. Use of vector according to any one of preceding claims 11-15 to produce transgenic plants containing the nucleic acid according to any one of preceding claims 1-10, under the control of specific regulatory sequences, preferably in selected organs of plants.

17. Use of vector according to claim 16 wherein said transgenic plant belongs to pepper, eggplant, soybean, grape, melon, rice, carrot, spinach, citrus, pomaceae or ornamental species.

18. Transgenic plant obtainable by the use of transforming vector according to any one of the preceding claims 11-15.

19. Transgenic plant according to claim 18 belonging to pepper, eggplant, soybean, grape, melon, rice, carrot, spinach, citrus, pomaceae and ornamental species.

20. Protein, or portions thereof, which, if altered, is responsible for the light hypersensitive phenotype in *Solanum lycopersicum* plants.

21. A protein according to claim 20 homologous with at least one of the *Arabidopsis* COP/DET/FUS family proteins, whose alteration is responsible for a light hypersensitive phenotype.

22. Protein according to claim 21 comprising the amino acid sequence of SEQ ID No. 2, or portions thereof.

23. Protein according to claim 22 comprising an alteration to the amino acid sequence of SEQ ID No. 2, or portions thereof, which are able to induce a light hypersensitive phenotype.

24. Protein according to claim 23 comprising at least an alteration in the SEQ ID No. 1 C-terminal portion.

25. Protein according to claim 24 wherein said alteration in the C-terminal portion comprises a substitution of proline at 498 position.

26. Protein according to claim 25 wherein said substitution comprises a serine as a substitute for proline.

27. Protein according to claim 24 wherein said alteration in the C-terminal portion comprises a deletion of at least one amino acid in the second NLS domain.

28. Protein according to claim 27 wherein said deletion comprises at least three amino acids.

29. Protein according to claim 28 wherein said deletion comprising at least three amino acids is from aa. 478 to aa. 480.

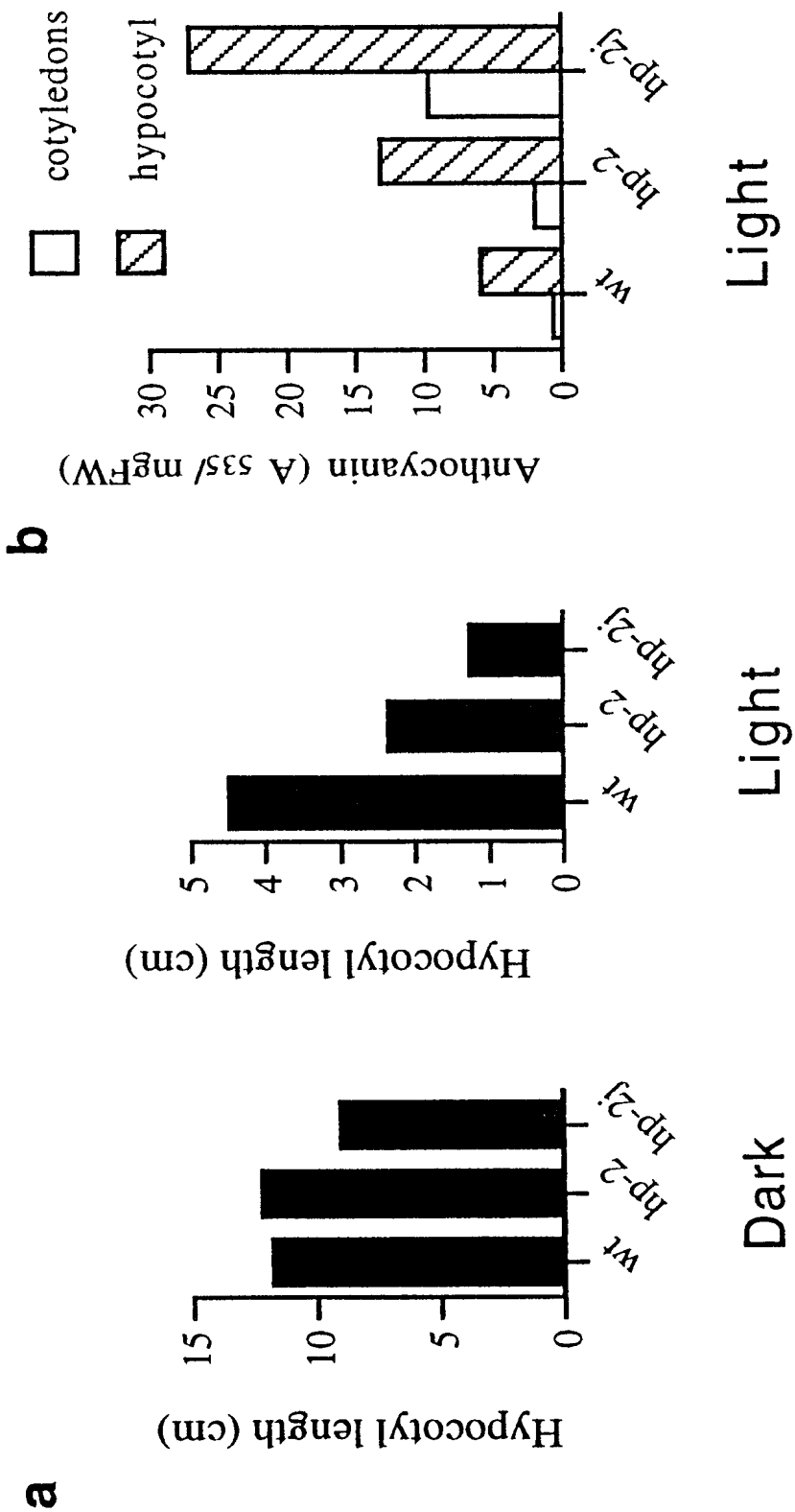

a
Hypocotyl length (cm)
0
5
10
15
wt
hp-2
hp-2j
Dark
Hypocotyl length (cm)
0
1
2
3
4
5
wt
hp-2
hp-2j
Light
b
Anthocyanin (A 535/ mgFW)
0
5
10
15
20
25
30
wt
hp-2
hp-2j
Light
cotyledons
hypocotyl

Fig. 1

```
   1  cttccctctt agactttatc gatcctaatt cgagccctcc ttttttcaat
  51  caattatcaa tttagtccta ctgcgatttt gatatgtatg attcacaatt
 101  ttaatgctgc tgaaagcaat tatataaaag ctgaaacatt ttgcactgAT
 151  GTTCAAAACT AACAATGTTA CCGCCAGGCT TTTTGAGCGC CAGATTTGCA
 201  CCCCTGCTCC TGGCACCAGC ATCCATCGTG CCAGAAGATT TTATGAGAAC
 251  GTTGTACCAA GTTATACCAT ATACGATGTT GAATGTCCCG ACCATTCATT
 301  TCGCAAGTTC ACGGATGACG GTCTATATTT TGTAAGTTTC AGCCGAAACC
 351  ATCAGGATCT GGTTGTTTAT AGACCAACAT GGCTGACATT TTCCTGCAAA
 401  GAAGAAGATT GTGATACTCA TGATCTTCCT TTGAAAGCTA GAAAGTTTGA
 451  GAGCTTCTTC ACACAGTTGT ACAGTGTTAC TCTTGCTTCT AGTGGGGAAC
 501  TTATATGCAA AGATTTCTTT CTCTATATGG AGAGCAACCA ATTTGGACTC
 551  TTTGCAACTT CAACTGCACA AATTCATGAT GCACCTCCTA CTGGAGGGGC
 601  AATTCAGGGA GTCCCTTCAG TTGAAAAAAT AACTTTCCAC CTTTTGAGGT
 651  TGGTGGATGG AGCTATACTT GACGAAAGGG TTTTCCACAA TGATTATGTT
 701  AATTTGGCAC ATAGCATTGG TGCTTTCTTG TATGATGATT TGCTTGCTAT
 751  AGTGTCTCTT CGTTATCAAA GAATACACAT CCTTCAGATC AGAGATTCTG
 801  GAGATCTTGT TGATGTACGA GCAATTGGGG AATTCTGCCG TGAAGATGAT
 851  GAACTTTTTC TCAATTCCAA TTCCCAGGTG CTTGTAAATC ATGTTGGAAA
 901  TGGTTTTCAT CATAGTCTGC CTCAATCAGA AACTTCTTTC CTGAGCGGTA
 951  TAAAGCAACG GCTGCTTTCA TATATATTTC GAGGTATATG GAATGAAGCT
1001  GACCAAACCA TGAGAGTGCA GTGCCTGAAG AAGAAGTTTT ACTTCCACTT
1051  TCAAGATTAC ATTGACTTGA TTATCTGGAA GGTGCAGTTT TTGGACCGAC
1101  ATCACCTGTT GATCAAGTTT GGCAGTGTTG ATGGTGGGGT ATCCCGAAAT
1151  GCTGACATCC ATCCTTCTTT TTTTGCTGTT TACAATATGG AGACTACTGA
1201  AATTGTTGCA TTTTATCAGA ACTCAGCCGA TGAGCTTTAT TTCTTGTTCG
1251  AGCTGTTCAG CGACCATTTT CACGTTTCAT CCAAAAGTTC ATTACATATG
1301  AACTTCATGT CCTCACACTC AAACAACATC CACGCCCTCG AGCAACTAAG
1351  GTGTACAAAG AACAAAGCAA CCAATTTCTC TCAATTTGTT AAGAAAATGA
1401  TGGCTTCCTT GCCTTGTAGT TGTCAGTCTC AGAGTCCTTC CCCATATTTT
1451  GACCAATCTC TCTTCAGGTT TGACGAGAAG CTTATTTCAG CTATTGACCG
1501  CCATAGACAG TCTACTGACC ATCCAATCAA ATTCATTTCT AGAAGACAAC
1551  CCAATATCCT GAAATTCAAA ATGAAGCCAG GACCTGAAGC TGGCAGCACA
1601  GATGGGCGAA CTAAGAAGAT CTGTTCCTTC CTCTTCCACC CAATATTACC
1651  CCTTGCACTT TCTGTTCAAC AAACCTTGTT TCTGCAGGCA TCAGTTGTAA
1701  ATATCCATTT TCGTCGATAA tgtaaaaact taatttatat gttaccgatt
1751  tgtttataaa tttctctaat aacctctaga ttgaaatcaa cctagaaatc
```

Fig. 2

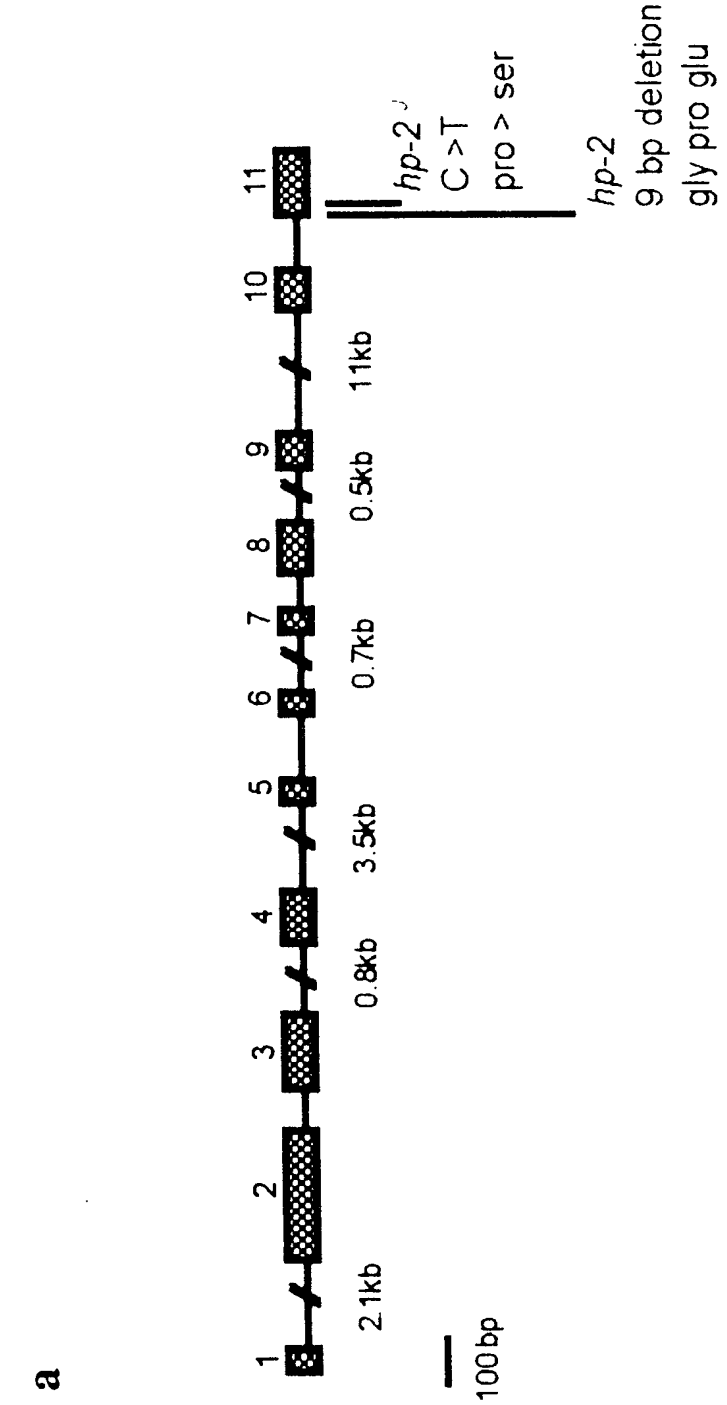
```
1801  acaaattcat cataacagac ccgtagatgc tagtgtcttt gacttctaca
1851  ttttctttgt tacaagaatc aaacaaatgc ttgatagagt gccaagacgg
1901  ttagtatggg tataaggatt agttcttctg taagtttttg ttacagcttc
1951  tcttctaatt aattgatgta cattcagatg tttaaaaaaa aaaaaaaaaa
```

Fig. 2

```
  1  MFKTNNVTAR LFERQICTPA PGTSIHRARR FYENVVPSYT IYDVECPDHS
 51  FRKFTDDGLY FVSFSRNHQD LVVYRPTWLT FSCKEEDCDT HDLPLKARKF
101  ESFFTQLYSV TLASSGELIC KDFFLYMESN QFGLFATSTA QIHDAPPTGG
151  AIQGVPSVEK ITFHLLRLVD GAILDERVFH NDYVNLAHSI GAFLYDDLLA
201  IVSLRYQRIH ILQIRDSGDL VDVRAIGEFC REDDELFLNS NSQVLVNHVG
251  NGFHHSLPQS ETSFLSGIKQ RLLSYIFRGI WNEADQTMRV QCLKKKFYFH
301  FQDYIDLIIW KVQFLDRHHL LIKFGSVDGG VSRNADIHPS FFAVYNMETT
351  EIVAFYQNSA DELYFLFELF SDHFHVSSKS SLHMNFMSSH SNNIHALEQL
401  RCTKNKATNF SQFVKKMMAS LPCSCQSQSP SPYFDQSLFR FDEKLISAID
451  RHRQSTDHPI KFISRRQPNI LKFKMKPGPE AGSTDGRTKK ICSFLFHPIL
501  PLALSVQQTL FLQASVVNIH FRR
```

Fig. 3

Fig. 4


a
1
2
3
4
5
6
7
8
9
10
11
2.1kb
0.8kb
3.5kb
0.7kb
0.5kb
11kb
100 bp
hp-2
C >T
pro > ser
hp-2
9 bp deletion
gly pro glu
b
wild type
... AAG CCA G GTACGG....TATCAG GA CCT GAA GCT GGG ...
K P G P E A G
hp-2
... AAG CCA G GTACGG....TATCTG GA CCT GAAG CT GGG ...
K P A G

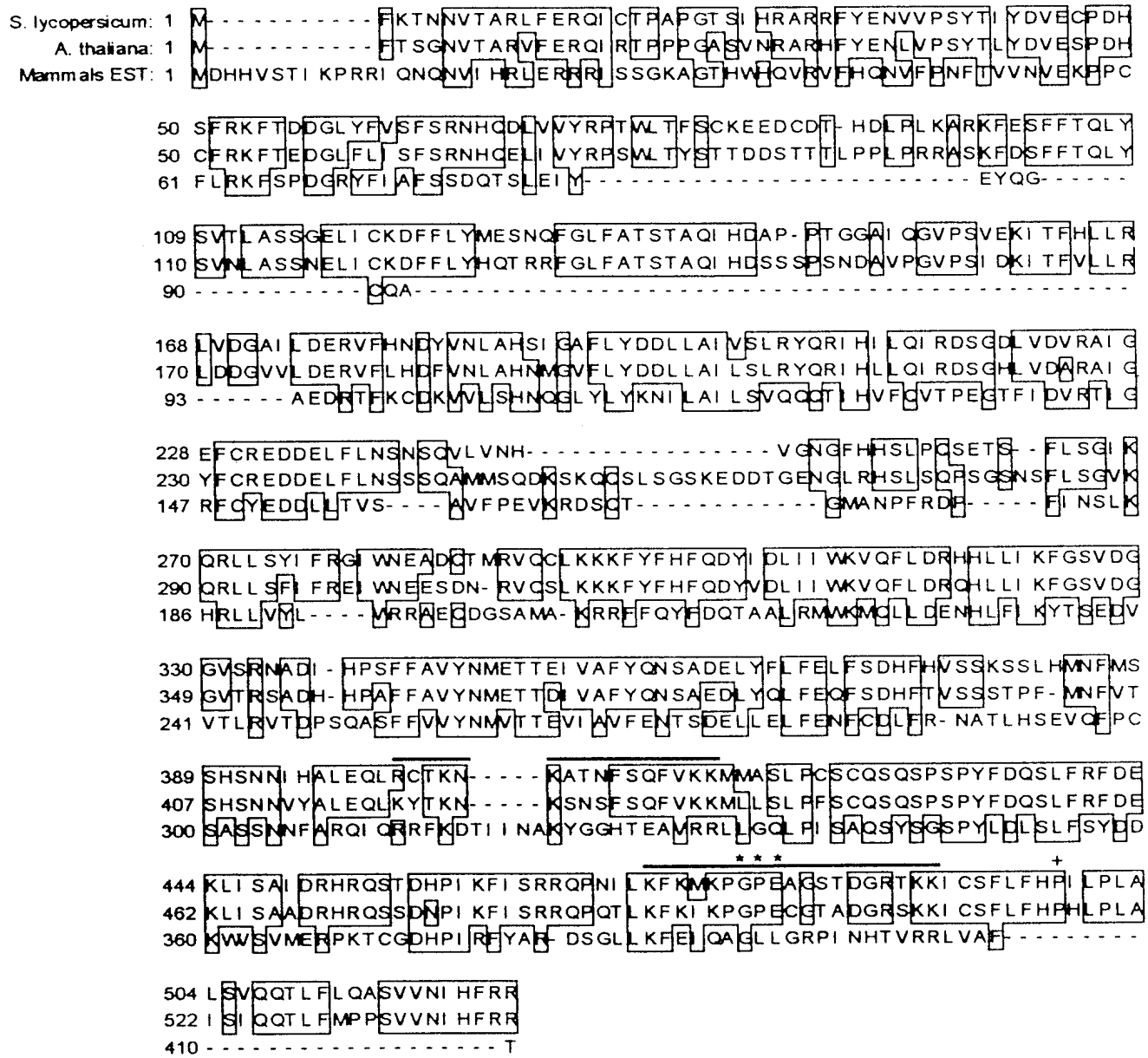
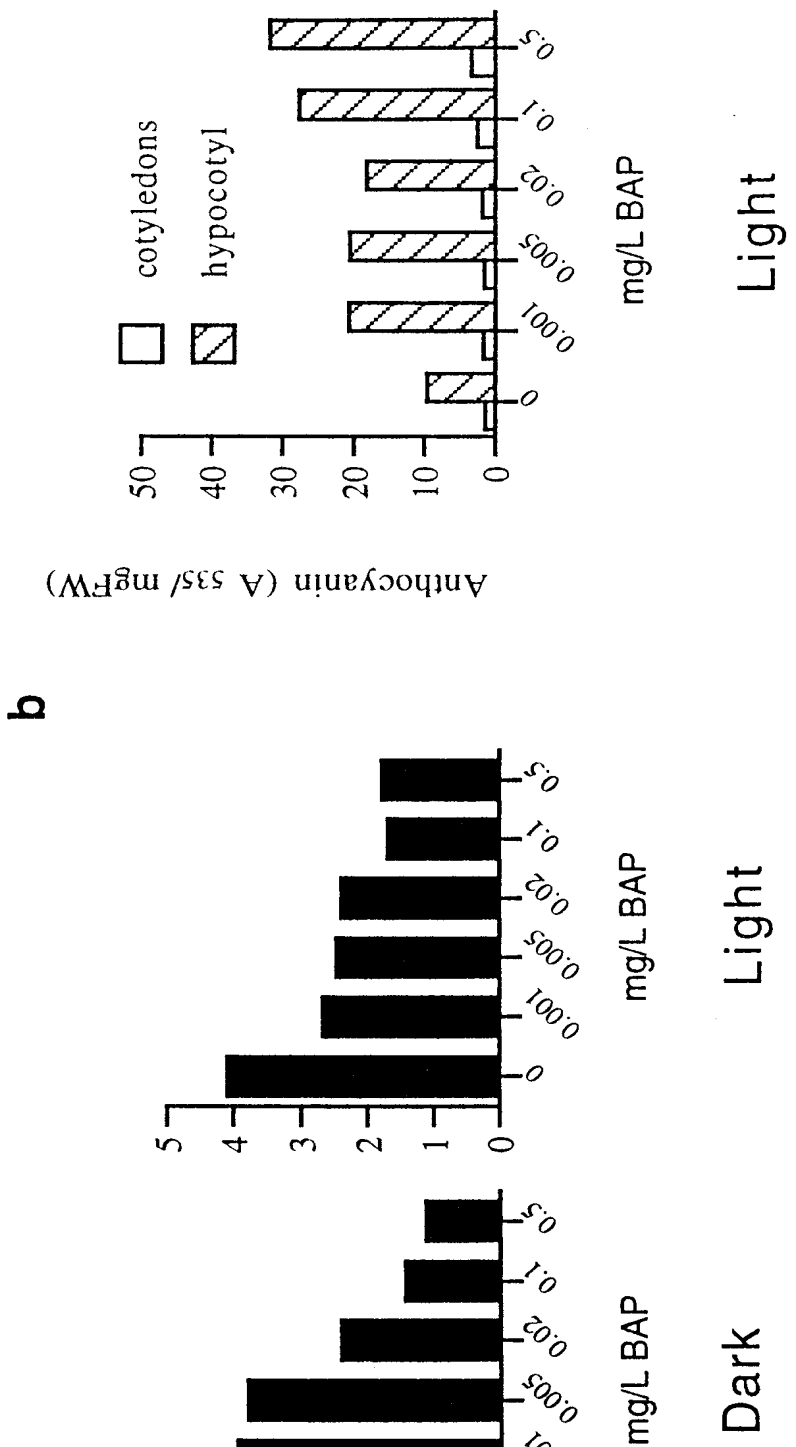
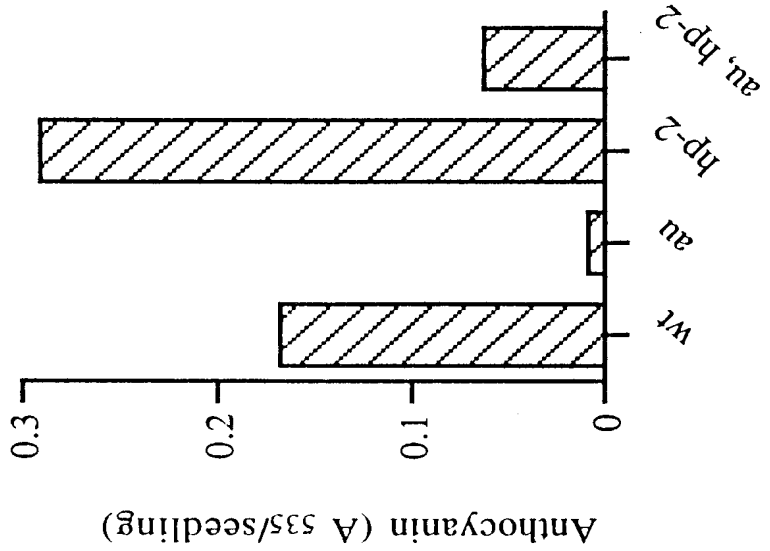


Fig. 5

Fig. 6


a
Hypocotyl length (cm)
15
10
5
0
0
0.001
0.005
0.02
0.1
0.5
mg/L BAP
Dark
5
4
3
2
1
0
0
0.001
0.005
0.02
0.1
0.5
mg/L BAP
Light
b
Anthocyanin (A 535/ mgFW)
cotyledons
hypocotyl
50
40
30
20
10
0
0
0.001
0.005
0.02
0.1
0.5
mg/L BAP
Light


Anthocyanin (A 535/seedling)
0
0.1
0.2
0.3
wt
au
hp-2
au, hp-2

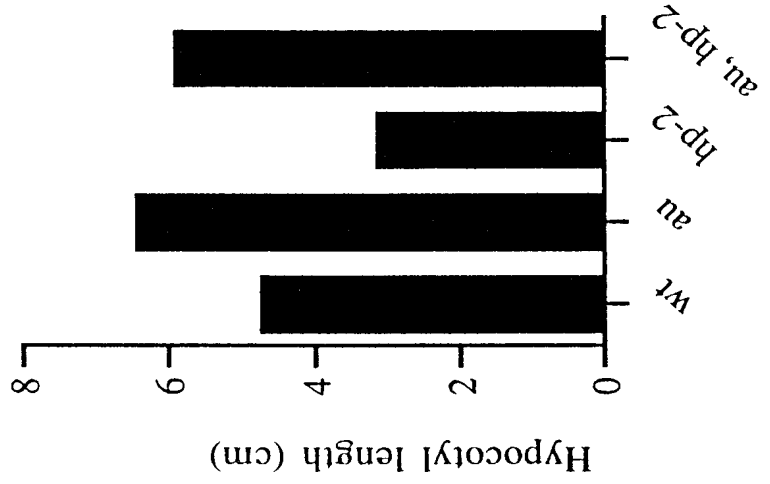

Hypocotyl length (cm)
0
2
4
6
8
wt
au
hp-2
au, hp-2

Fig. 7

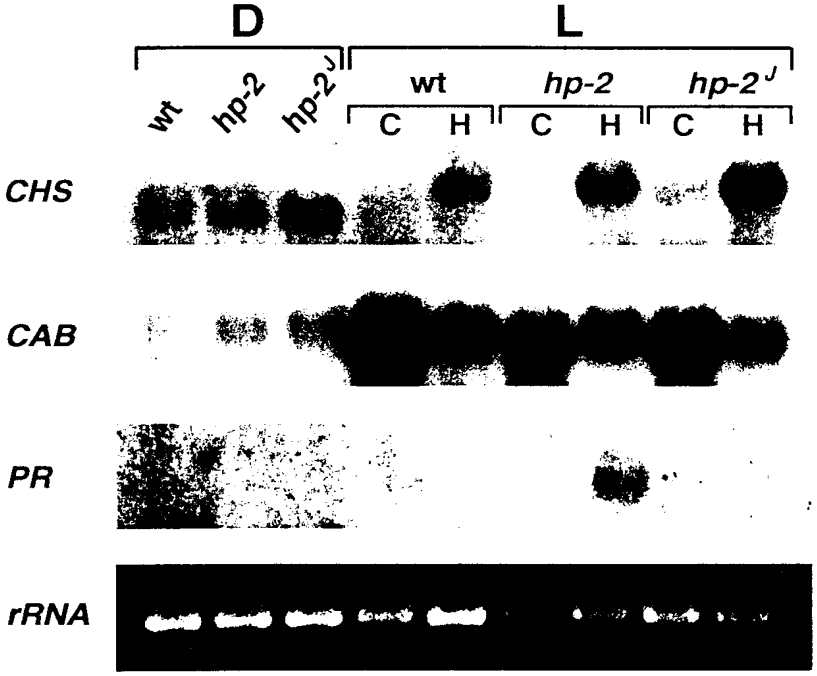
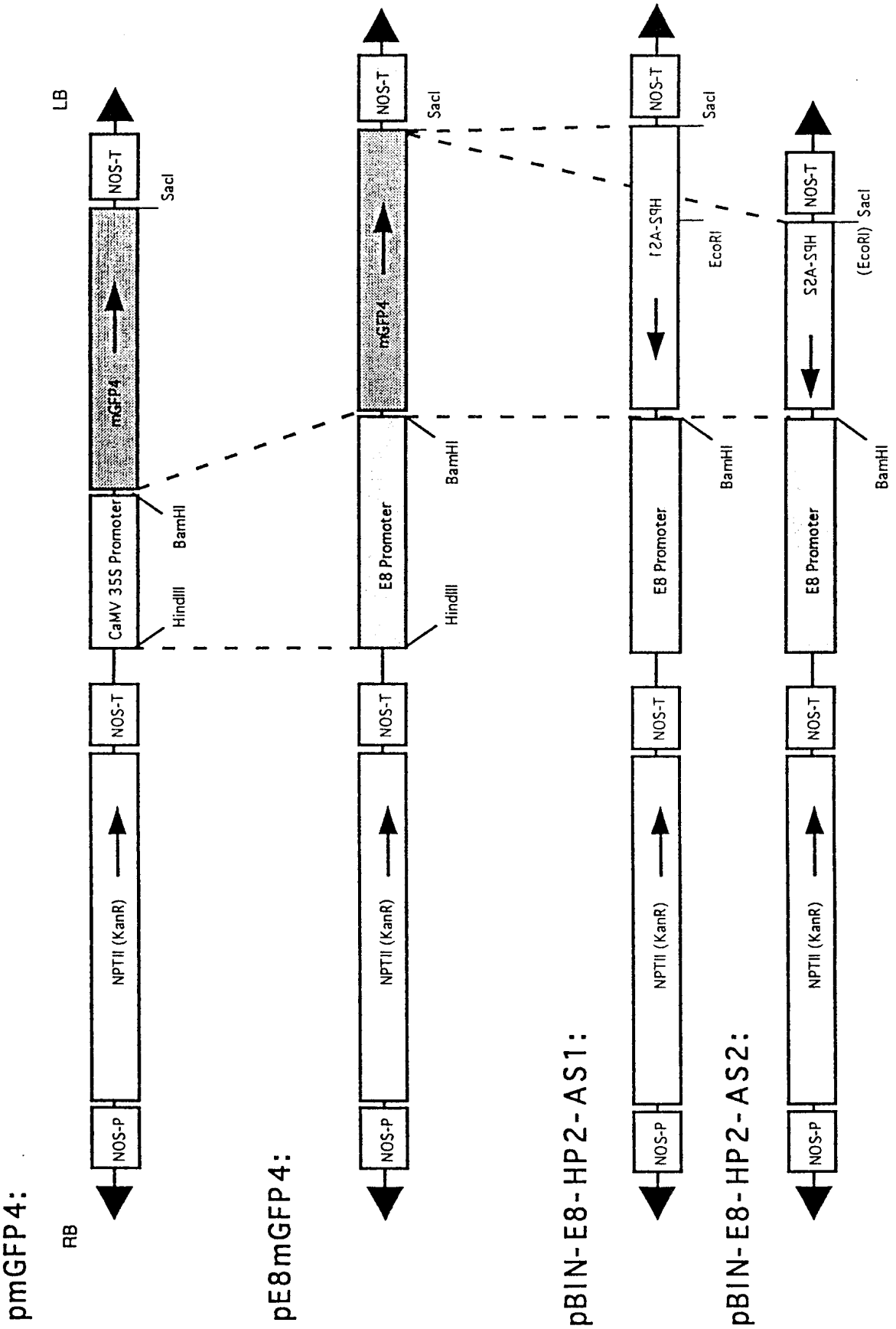


Fig. 8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LB
RB
NOS-P
NPTII (KanR)
NOS-T
CaMV 35S Promoter
HindIII
BamHI
mGFP4
SacI
NOS-T
pE8mGFP4:
NOS-P
NPTII (KanR)
NOS-T
E8 Promoter
HindIII
BamHI
mGFP4
SacI
NOS-T
pBIN-E8-HP2-AS1:
NOS-P
NPTII (KanR)
NOS-T
E8 Promoter
BamHI
f2A-S9H
EcoRI
SacI
NOS-T
pBIN-E8-HP2-AS2:
NOS-P
NPTII (KanR)
NOS-T
E8 Promoter
BamHI
S2A-S9H
(EcoRI)
SacI
NOS-T

Fig. 9

INTERNATIONAL SEARCH REPORT

Inter ›nal Application No
PCT/IT 98/00350

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/29 C12N15/82 C07K14/415 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FRAY R G ET AL: "CONSTITUTIVE EXPRESSION OF A FRUIT PHYTOENE SYNTHASE GENE IN TRANSGENIC TOMATOES CAUSES DWARFISM BY REDIRECTING METABOLITES FROM THE GIBBERELLIN PATHWAY" PLANT JOURNAL, vol. 8, no. 5, November 1995, pages 693-701, XP002043131 see the whole document ---	1,11
X	TANKSLEY, S.D., ET AL.: "High density molecular linkage maps of the tomato and poato genomes" GENETICS, vol. 132, December 1992, pages 1141-1160, XP002097980 see page 1142, left-hand column; figure 1 --- -/--	1-3

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 April 1999	16/04/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Maddox, A

5

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	GANAL, M.W., ET AL.: "CT151.KS Tomato leaf cDNA from cv. VFNT cherry Lycopersicon esculentum cDNA clone CT151" EMBL ACCESSION NO. AA824865, 20 February 1998, XP002097199 see the whole document	1-7
Y	VAN TUINEN, A., ET AL.: "The mapping of phytochrome genes and photomorphogenetic mutants of tomato" THEOR APPL GENET, vol. 94, 1997, pages 115-122, XP002097981 see page 117 - page 118	1-10
Y	KERCKHOFFS, L.H.J., ET AL.: "Photocontrol of anthocyanin biosynthesis in tomato" J PLANT RES, vol. 110, 1997, pages 141-149, XP002097982 see page 146, right-hand column - page 147, left-hand column, line 5	1-10
A	KERCKHOFFS, L.H.J., ET AL.: "Physiological characterization of exaggerated photoresponse mutants of tomato" J. PLANT PHYSIOL., vol. 150, 1997, pages 578-587, XP002097983 see the whole document	1-29
A	YEN H. C., ET AL.: "The tomato high-pigment (hp) locus maps to chromosome 2 and influences plastome copy number and fruit quality" THEORETICAL AND APPLIED GENETICS, (NOV 1997) VOL. 95, NO. 7, PP.1069-1079. , XP002097198 see the whole document	1
A	PEPPER, A., ET AL.: "DET1, a negative regulator of light-mediated development and gene expression in Arabidopsis, encodes a novel nuclear-localized protein" CELL, vol. 78, 1994, pages 109-116, XP002097200 see the whole document & EMBL ACCESSION NO. L33695,20 July 1994,	1-10, 20-29
A	BOYLAN, M.T., ET AL.: "Oat phytochrome is biologically active in transgenic tomatoes" THE PLANT CELL, vol. 1, 1989, pages 765-773, XP002097201 see the whole document	11-19

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/IT 98/00350

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	MCNELLIS, T.W., ET AL.: "Expression of an N-terminal fragment of COP1 confers a dominant-negative effect on light-regulated " THE PLANT CELL, vol. 8, September 1996, pages 14901-1503, XP002097202 see the whole document	11-19
A	MCNELLIS, T.W., ET AL.: "Overexpression of Arabidopsis COP1 results in partial suppression of light-mediated development: evidence for a light-inactivatable repressor of photomorphogenesis" THE PLANT CELL, vol. 6, 1994, pages 1391-1400, XP002097203 see the whole document	11-19
A	WO 97 20941 A (UNIV KINGSTON ;KO KENTON (CA); KO ZDENKA W (CA); LABATE CARLOS (BR) 12 June 1997 see page 42, line 5 - line 28	11-19
A	WO 97 39112 A (LI JIANMING ;CHORY JOANNE (US); SALK INST FOR BIOLOGICAL STUDI (US) 23 October 1997 see the whole document	11-19
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